filed on September 4, 2001 does not fully comply with the requirements of 37 CFR 1.98 because the titles of the Journal articles are missing, (3) the information disclosure statement filed on May 14, 2001 has been lost and (4) several journal articles in the information disclosure statement filed on June 12, 2001 will not be initialed because they are duplicates of journal articles cited in the IDS filed on June 12, 2000 which have already been initialed.

In response, Applicants submit herewith an amended PTO Form 1449 corresponding to the PTO Form 1449 filed on August 31, 2001 and received by the Unites States Patent and Trademark Office on September 4, 2001 PTO Form 1449, in which the journal article titles are included. In addition, Applicants submit second supplemental Information Disclosure Statement and PTO Form 1449 for new references cited in the Declaration submitted herewith. While the Examiner hsa indicated that the IDS filed on May 14, 2001, can not be located, Applicants do not know of any IDS filed on that date. The only papers which Applicants believe were filed on May 10, 2001 and received by the United States Patent and Trademark Office on May 14, 2001, are a claim for priority and a Preliminary Amendment to amend the specification for the claim of priority.

Applicants note that the drawings have been considered acceptable by the Examiner.

II. Claim and Specification Objections

The Examiner has objected to claims 4-6, 12-31 and 47-49 under 37 C.F.R. 1.75 (c) as being in improper form for having multiple dependent claims depend on other multiple dependent claim. In addition claims 1, 7, and 11 have been objected to for minor informalities.

In response, Applicants have amended claims 4-6, 12-31 and 47-49 to correct

improper multiple dependencies. Claim 1 and 7 have been amended to change the term "intron/s" to "introns." Claim 11 has been amended to correct improper Markush language. Therefore, Applicants respectfully request withdrawal of the objections to claims 1, 4-7, 11-31 and 47-49.

The Examiner also has objected to the abstract for containing improper terms and for being more than 150 words in length. In response, Applicants have amended and shortened the abstract. Applicants believe the abstract is now in proper condition and respectfully request withdrawal of the objection.

The Examiner has further objected to the specification for containing reference to hyperlink and/or other form of browser-executable code. In response, Applicants have amended the specification to delete reference to any hyperlink and/or other form of browser-executable code and respectfully request withdrawal of the objection.

III. Rejections Under 35 U.S.C. § 112

The Examiner has rejected claims 1-3 and 7-11 under 35 U.S.C. 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors had possession of the claimed invention. The Examiner contends that these claims are readable on a genus of a *cis*-acting nucleotide sequence, which is capable of rendering the removal of introns from a precursor transcript, dependent upon activation of a *trans*-acting factor. However, according to the Examiner, the specification does not provide an adequate written description of a "genus" of *cis*-acting nucleotide sequence, for example, any description of specific biochemical or molecule structure (e.g., nucleotide sequence) of a representative number of species of such *cis*-acting nucleotide sequences. The Examiner thus states that the disclosure provides sufficient support only for SEQ ID NO: 1 and 2, but not for the "generic" *cis*-acting nucleotide sequences.

In response, Applicants submit herewith a declaration by Prof. Kaempfer, as well as Exhibits A-F referenced by the declaration, which shows in accordance with the present invention two novel *cis*-acting nucleotide sequences having splicing regulatory activity. One element, derived from the human β-globin gene, has been shown by Applicants to be a PKR activator and an inducer of efficient and PKR-dependent splicing. The second novel element found by the Applicants, is derived from the IFN-γ gene. Applicants have shown that this *cis*-acting nucleotide sequence, when inserted into the 3'UTR region of human TNF-β gene, significantly activates PKR and induces an efficient and PKR-dependent splicing.

In addition to the declaration, Applicants assert that a "generic" sequence is not essential for the written description because these elements are described by the specification using functional characteristics, *i.e.* as being capable of regulating splicing dependent on PKR and sensitive to 2-AP and being capable of activating PKR. The specification further provides methods of evaluating potential sequences having this function in the Examples. This disclosure, together with the attached Declaration show that adequate written description for the genus of *cis*-acting nucleotide sequences of the claims is found in the specification and that Applicants were in possession of the claimed invention at the time of filing.

The Examiner further contends, with respect to using a trans-acting factor in claims 1-3 and 7-11, that the specification only supports the use of PKR as the *trans*-acting factor and not any other RNA-activated protein kinases capable of phosphorylating the alpha-subunit of eIF2. In response, Applicants have amended claims 1 and 7 so as to be restricted to PKR and have cancelled claim 2.

The Examiner has rejected claims 2, 8-9 and 11 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject

matter which the applicant regards as the invention. Specifically, the Examiner alleges that the claims are improperly dependent and for using ambiguous language. In response to this rejection, these claims have been amended to correct the improper dependencies and remove or amend the ambiguous language.

IV. Rejections Under 35 U.S.C. § 102(b)

The Examiner rejects claim 1 under 35 U.S.C. 102 (b) as being anticipated by Pennica *et al.*, which discloses the nucleotide sequence of the human TNF-α gene including SEQ ID NO: 1 and 2.

Applicants respectfully disagree. Pennica *et al.* discloses the nucleotide and amino acid sequence of the full length human TNF-α gene, that includes SEQ ID NO:1 and 2 of the present application. Fig. 1, page 725 of Pennica *et al.* gives the entire TNF-α gene sequence. However, the present claims define a *cis*-acting element. The disclosure of the entire TNF-α gene sequence dose not imply or even hint at the existence of a *cis*-acting element within said gene, neither does it identify any such element, which forms the basis for claim 1. Still further, the skilled person, when considering this publication, cannot learn about any *cis*-acting element which has splicing regulatory properties, and not about any of the other *cis*-acting elements found recently by Applicants and further described in the Declaration submitted herewith. Therefore, Pennica *et al.* does not anticipate the claims of the present invention. Nonetheless, to expedite examination, claim 1 has been amended to specify that the *cis*-acting element of the invention does not comprise a full-length coding region.

The Examiner further rejects claims 1-3 and 7-10 under 35 U.S.C. 102 (b) as being anticipated by Jarrous *et al.* The Examiner contends that Jarrous *et al.* discloses a vector comprising

the TNF-α gene including the 3' untranslated region, which reads on a *cis*-acting nucleotide sequence of the present application, and further teaches that the trans-acting factor for the sequence is PKR.

Applicants respectfully disagree. As stated by the Examiner, Jarrous *et al.* discloses a vector (phTNF-α) comprising the full length TNF-α gene, including the upstream regulatory sequences (and not the 3'-UTR, see page 2815, first column, lines 17-18), a carrier (salmon sperm DNA) and a host BHK-21 cell line transformed with said vector. As shown by Figure 4 (see page 2817, second column), transfection of these cells has shown that processing of precursor RNA transcribed from an exogenous TNF-α gene is sensitive to 2-AP.

By contrast, the present invention provides for vectors comprising a defined *cis*-acting nucleotide sequence having specific properties of rendering the removal of introns from a precursor transcript encoded by any gene (for example, TNF-β, Fig 7A-7C), dependent upon activation of a *trans*-acting factor, which is an RNA-activated protein kinase capable of phosphorylating eIF2α, specifically, PKR.

In general, Jarrous *et al.* discloses that 2-AP inhibits the expression of TNF-α mRNA elicited by different inducers, but does not have an effect on the expression of IL-1β (Figure 1). This document further shows (Figure 2) that inhibition of TNF-α expression by 2-AP, and the concomitant sharp rise in the level of precursor transcripts results from a failure of precursors to be spliced into mRNA. In Figure 3, Jarrous *et al.* compares between pentoxifylline and 2-AP, and shows that pentoxifylline inhibits the synthesis of a precursor RNA, rather an effect on the splicing. Figure 5 shows that 2-AP elicits accumulation of unstable, functional TNF-α transcript. Still further, Figure 6 shows that 2-AP inhibits TNF-α gene expression through a mechanism distinct from that used by protein kinase inhibitors of transcription. In contrast, Figure 7 shows that in HL-60 cells, 2-

AP blocks TNF-α gene transcription. However, in PBMC, increasing concentrations of 2-AP induced decline in the levels of spliced mRNA concomitant with a rise in the level of precursor transcript (Figure 8).

Thus, Jarrous *et al.* does not describe or even hint at the novel splicing control system disclosed by the present invention which involves the particular concomitant interaction of both a *cis*-acting element and a particular *trans*-acting element, as defined by claim 1. Contrary to the Examiner's stated conclusion mentioned above: " ...and further teaches (Jarrous *et al.*) that the *trans*-acting factor for the sequence is PKR", this document does not show any functional connection between a particular sequence and this particular kinase.

The Examiner states that Jarrous *et al.* (page 2820, column 1, lines 5-24) exemplifies a method of regulating gene expression at the mRNA level by transforming a host cell with a vector comprising the TNF-α gene, including the 3' untranslated region, wherein the activity of the RNA activated eIF2 alphakinase in the host cells is modulated by the use of 2-AP. Applicants wish to point out that this paragraph only shows comparison of the responses of the expression of TNF-α mRNA and precursor transcripts to increasing concentrations of 2-AP in HL-60 cells and PBMC, using a probe which detects splicing at different sites within human TNF-α primary transcripts. The results of these experiments confirmed that in HL-60 cells, 2-AP inhibits transcription of TNF-α, whereas in PBMC, 2-AP inhibits the splicing of TNF-α transcript at multiple sites, including intron 2 and intron 3 splice junctions. Moreover, Jarrous *et al.* (page 2820, column 1, lines 5-24), although describing use of 2-AP, does not disclose use of a DNA construct which has been modified by the inclusion of a *cis*-acting nucleotide sequence having the properties defined in claim 1. Instead, expression of the native TNF-α gene inside human cells is followed under that passage. Jarrous *et*

al. does not disclose the existence of such *cis*-acting element, needless to say there is nothing in this document to have taught the man skilled in the art to use it as regulatory element for controlling the expression of different genes. Furthermore, this paragraph, as well as the complete Jarrous *et al.* document does not disclose or even suggest the existence of a particular *cis*-acting element which in response to a particular *trans*-acting element, regulates splicing.

The Examiner further stated that Jarrous *et al.* clearly indicates that "Most likely, regulation by 2-AP is mediated by a particular sequence within the TNF-α primary transcript to produce general inhibition of the splicing of this transcript" (cf. Jarrous *et al* page 2821 column 1, lines 38-40). This sentence is totally unsupported by the results shown in Jarrous *et al*. No sequence is described, nor is its likely or, even speculative, location discussed.

The Examiner further states that Jarrous *et al*: teach "deletion of a particular sequence from the TNF-α gene renders splicing of the encoded precursor transcripts resistant to inhibition by 2-AP, while introduction of said sequence into the TNF-β gene shifts the inhibitory effect of 2-AP on the TNF-β gene expression from transcription to slicing" (cf. page 2821 column 1, lines 45-51), briefly reported by the authors which are the inventors (unpublished data)". Although this citation uses the word "sequence", it does not specify the nature or location of such sequence, neither does it even hint at the involvement of a *trans*-acting factor as defined in claim 1. A person of skill in the art would not have been able to know from this passage whether the sequence is nearly as long as the entire gene, or merely a few nucleotides long, or in between in length. Furthermore, for the invention as defined in the claims, it would be necessary that the sequence should not contain TNF-α protein-encoding sequence that would disturb expression of the desired gene, or be too long to be amenable to facile insertion without disturbing expression of the desired gene. Such knowledge is

not disclosed in Jarrous *et al.*, which reports only an inhibitory effect of 2-AP but not the properties defined in claim 1.

Jarrous et al., show that splicing of human TNF-α mRNA is inhibited by 2-AP but this work neither identified an RNA element nor did it link PKR to splicing of this mRNA. The invention, but not Jarrous et al., 1996, shows that a fragment of the TNF-α gene activates PKR at the RNA level and that a fragment of the TNF-α gene, when inserted into the TNF-β gene, renders splicing of that gene dependent on the activation of PKR. In doing so, the invention relies not only on the inhibitory effect of 2-AP, which, as will be seen below, is not specific for PKR, but directly on the inhibitory effect of a dominant-negative mutant of PKR, (*e.g.* PKRΔ6), which by forming heterodimers with wild type PKR blocks its activation and activity. The specification indicates that the effect of overexpressing wild type PKR, is to strongly stimulate the conversion of unspliced TNF-α pre-mRNA into the spliced mRNA form. Thus, the invention shows the involvement of PKR in splicing of TNF-α mRNA and teaches a *cis*-acting PKR-activator element in the TNF-α 3'-UTR that renders splicing both dependent on PKR and responsive to changes in the intracellular level of PKR. Jarrous et al. did not do any of these things.

Still further, the Examiner indicates that: "the state of the art teaches that two types of kinase (PKR and heme) that are capable of phosphorylating the alpha-subunit of eIF2 (Jarrous *et al.*, page 2814)". Applicants assert that the family of eIF2α kinases comprises not only PKR but also other members, e.g., the heme-controlled eIF2α kinase HCR [Farrell et al., 1977, attached as **Exhibit E**] and the eIF2α kinase PERK (also termed PEK) which is activated during the unfolded protein response [Ron and Harding, 2000, **Exhibit F**]. All the eIF2α kinases are similarly

characterized by their ATP binding site in the C-terminal kinase domain of the protein [Farrell et al., 1977; Ron and Harding, 2000; Ben-Asouli et al., 2002 **Exhibits E, F and C** respectively], which engenders sensitivity to 2-AP as was demonstrated for HCR and PKR. Moreover, although sensitivity to 2-AP is an outstanding property of eIF2α kinases, it is also observed for a number of other, less well-characterized kinases, be it to a lesser extent. Therefore, the observation of Jarrous et al. that splicing of TNF-α mRNA is sensitive to 2-AP left open the nature of the possible step that might be inhibited, whether involving an eIF2α kinase, another protein kinase, or no kinase. Work on the activation of PKR by 2-APRE and analysis of the effect of wild type and mutant forms of PKR on TNF-α mRNA splicing in cells were needed to demonstrate the involvement of PKR and 2-APRE, as Applicants have done in the present application.

Uniquely among the eIF2α kinases, PKR requires RNA for its activation and prior to the invention it was generally accepted that (linear) double-stranded RNA is needed. The finding that the single-stranded, only partially folded 2-APRE does not fulfill the criteria of a typical double-stranded RNA shows the novelty of activation of PKR by this element.

Thus, Jarrous *et al.*, although indicating that 2-AP is an inhibitor of PKR and heme kinases, and that splicing of TNF-α is inhibited by 2-AP, does not describe or show the existence of a *cis*-acting sequence which specifically responds to a particular *trans*-acting factor which is the PKR. Even with knowledge of the sequence of the complete human TNF-α gene supplied by Pennica et al. in 1984, the fact is that until the invention, many years later, no one had reported on the role of an element within this gene that renders splicing dependent on PKR. It was left to the inventors to do so. This fact illustrates that one skilled in the art would not be able by looking at the gene sequence to predict that an element with the properties of the 2-APRE is contained therein.

Dissection of the mechanism of TNF-α mRNA expression, demonstration of the activation of PKR by an RNA fragment encoded by the gene, direct demonstration of the involvement of PKR in splicing of the mRNA, mapping of the element to a portion of the 3'-UTR and analysis of its structure was needed to identify the 2-APRE and show its properties, as was done by the inventors.

In summary, the Jarrous *et al.* citation does nothing to advance the insight of a man of the art into the nature of the novel splicing control as disclosed in the specification and defined in the claims, that form the basis for the invention, based on a concretely defined novel *cis*-acting nucleotide sequence and a defined *trans*-acting element. Use of said *cis*-acting nucleotide sequences, whether derived from TNF-α (as shown by the present invention) or derived from human β-globin gene or IFN-γ (as shown by the declaration) in conjunction with said *trans*-acting factor is not disclosed or even hinted at in Jarrous *et al.* or in Pennica *et al.*

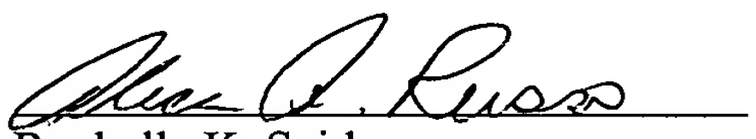
V. Conclusion:

In view of the foregoing comments and amendments, favorable consideration and allowance of all pending claims is earnestly solicited.

Applicants have enclosed the fee for a three-month extension of time as required under 37 C.F.R. §1.17(a)(3). Applicants do not believe that any additional fee is required for this filing. Nevertheless, the Commissioner is hereby authorized to charge any fees required for this submission not otherwise enclosed herewith to Deposit Account No. 02-4377. Two copies of this page are enclosed.

Respectfully submitted,

May 5, 2003



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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION

Please amend the Abstract as follows:

A *cis*-acting nucleotide sequence which is capable of rendering the removal of [intron/s] introns from a precursor transcript encoded by a gene, which gene harbors at least one such *cis*-acting nucleotide sequence, occurring during the production of mRNA of [said] a gene, dependent upon activation of a *trans*-acting factor. The *trans*-acting factor is an RNA-activated protein kinase which is capable of phosphorylating the α-subunit of eukaryotic initiation factor 2[,]. [or] The trans-acting factor is preferably, the RNA-activated protein kinase (PKR). The *cis*-acting nucleotide sequence can be derived from the 3' untranslated region of the human tumor necrosis factor α gene (TNF-α 3'-UTR)[. The *cis*-acting nucleotide sequence] and may comprise the nucleotide sequence [substantially] as denoted by SEQ ID NO:1[;] or biologically functional fragments, derivatives, mutants and homologues thereof. [of the nucleotide sequence substantially as denoted by SEQ ID NO:1; or a nucleotide sequence whose complementary nucleotide sequence hybridizes, under conditions which allow for such hybridization to occur, with the nucleotide sequences substantially as denoted by SEQ ID NO:1 or biologically functional fragments, derivatives, mutants and homologues of the nucleotide sequence substantially as denoted by SEQ ID NO:1.].

Please rewrite paragraph 1 , first line, page 31 of the specification as follows:

Example 4A

Splicing of TNF-α Precursor Transcripts Carrying TNF-β 3'-UTR Sequences is Insensitive to 2-AP

Please rewrite paragraph 3, page 32 of the specification as follows:

The structure of 3' UTR-αEP RNA transcript was analyzed by T1, U2 and V1 RNase sensitivity mapping (Fig. 5). 3'UTR-αEP RNA forms a stable, 5'-proximal 48-nt stem-loop containing 17 base pairs (DG= -59 kJ at 30°C). As calculated by the RNADraw

[{http://rnadraw.base8.se/)] and *mfold* algorithms [64][()replace by number]], this stem-loop structure persists in the longer EP-containing RNA fragments shown in Figure 4A.

IN THE CLAIMS

Please amend claims 1, 3-31 and 47-49 as follows:

1. A *cis*-acting nucleotide sequence which is capable of rendering the removal of [intron/s] introns from a precursor transcript encoded by [a] any gene, which gene harbors at least one such *cis*-acting nucleotide sequence, occurring during the production of mRNA of said gene, dependent upon activation of a *trans*-acting factor, said *trans*-acting factor being [an] the RNA-activated protein kinase (PKR) which is capable of phosphorylating the α-subunit of eukaryotic initiation factor 2, and wherein the cis-acting nucleotide sequence does not comprise a full-length coding region.

3. The [A] *cis*-acting nucleotide sequence according to claim 1 [or claim 2] derived from the 3' untranslated region of the human tumor necrosis factor α gene (TNF-α 3'-UTR).

4. The [A] *cis*-acting nucleotide sequence according to [any one of] claim[s 1 to] 3 which comprises:

   a) the nucleotide sequence substantially as denoted by SEQ ID NO:1; or
   b) biologically functional fragments, derivatives, mutants and homologues of the nucleotide sequence [substantially] as denoted by SEQ ID NO:1; or
   c) a nucleotide sequence whose complementary nucleotide sequence hybridizes, under conditions which allow for such hybridization to occur, with the nucleotide sequences of (a) or (b).

5. The [A] *cis*-acting nucleotide sequence according to claim 4, which comprises:

   a) the nucleotide sequence [substantially] as denoted by SEQ ID NO:2; or
   b) biologically functional fragments, derivatives, mutants and homologues of the nucleotide sequence [substantially] as denoted by SEQ ID NO:2; or
   c) a nucleotide sequence whose complementary nucleotide sequence hybridizes,

under conditions which allow for such hybridization to occur, with the nucleotide sequences of (a) or (b).

6. The [A] *cis*-acting nucleotide sequence according to [any one of] claim[s 1 to] 5 wherein said gene encodes a protein [is] selected from the group consisting of enzymes, hormones, growth factors, cytokines, structural proteins, [and] industrially applicable proteins, [or] agriculturally applicable proteins, [or is itself] a protein which is a therapeutic product, protein which is an agricultural product, [or] and a protein which is an industrially applicable product.

7. A DNA construct comprising:-

a)[-] a gene which contains at least one intron;

b) [-] a *cis*-acting nucleotide sequence which is capable of rendering the removal of [intron/s] introns from a precursor transcript encoded by said gene, which gene includes at least one such *cis*-acting nucleotide sequence, occurring during the production of mRNA of said gene, dependent upon activation of a *trans*-acting factor, wherein said *trans*-acting factor being [an] the RNA-activated protein kinase (PKR) which is capable of phosphorylating the α-subunit of eukaryotic initiation factor 2, operably linked to said gene; and

c) [-] optionally further comprising additional control, promoting and/or regulatory elements.

8. [A] The DNA construct according to claim 7 wherein said *cis*-acting nucleotide sequence comprises:

a) the nucleotide sequence [substantially] as denoted by SEQ ID NO:1; or

b) biologically functional fragments, derivatives, mutants and homologues of the nucleotide sequence [substantially] as denoted by SEQ ID NO:1; or

c) a nucleotide sequence whose complementary sequence hybridizes, under conditions which allow [for such] hybridization to occur, with the nucleotide sequences of (a) or (b).

9. [A] The DNA construct according to claim 7 wherein said *cis*-acting nucleotide sequence

comprises:

a) the nucleotide sequence [substantially] as denoted by SEQ ID NO:2; or

b) biologically functional fragments, derivatives, mutants and homologues of the nucleotide sequence [substantially] as denoted by SEQ ID NO:2; or

c) a nucleotide sequence whose complementary sequence hybridizes, under conditions which allow for such hybridization to occur, with the nucleotide sequences of (a) or (b).

10. A DNA construct according to any one of claims 7 to 9 wherein said control, promoting and/or regulatory elements are suitable transcription promoters, transcription enhancers and mRNA destabilizing elements.

11. [A] The DNA construct according to claim 7, wherein said gene which contains at least one intron, encodes a protein [is] selected from the group consisting of enzymes, hormones, growth factors, cytokines, structural proteins, [and] industrially applicable proteins, [or] agriculturally applicable proteins, [or is itself] a protein which is a therapeutic product, protein which is an agricultural product, [or] and a protein which is an industrially applicable product.

12. The [A] DNA construct according to [any one of] claim[s 7 to] 11 wherein said nucleotide sequence is contained within an exon of said gene.

13. The [A] DNA construct according to [any one of] claim[s 7 to] 11 wherein said nucleotide sequence is contained within an intron of said gene.

14. The [A] DNA construct according to [any one of] claim[s 7 to] 13 wherein said gene is the human TNF-α gene.

15. The [A] DNA construct according to claim 14 being the plasmid pTNF-α, in which said *cis*-acting element is contained within an exon of the human TNF-α gene.

16. The [A] DNA construct according to claim 15 being the plasmid pTNF-α(3'UTR-αEP).

17. The DNA construct [An expression vector] according to [any one of] claim[s] 7 [to 13]

wherein said gene is the human TNF-β gene.

18. The [A] DNA construct according to claim 17 in which said *cis*-acting element is contained within an exon of the human TNF-β gene.

19. The [A] DNA construct according to claim 18 being the plasmid pTNF-β(3'UTR-α).

20. The [A] DNA construct according to claim 18 being the plasmid pTNF-β(3'UTR-αEP).

21. The [A] DNA construct according to claim 14 in which said gene is the human TNF-α gene and said *cis*-acting element is contained within an intron of said gene.

22. The [A] DNA construct according to claim 21 being the plasmid pTNFα(Δ3'UTR)i3EP.

23. A vector comprising a *cis*-acting nucleotide sequence according to [any one of] claim[s] 1 [to 6] or a DNA construct according to [any one of] claim[s] 7 [to 22] and a suitable DNA carrier, capable of transfecting a host cell with said *cis*-acting nucleotide sequence.

24. The [A] vector according to claim 23 optionally further comprising additional expression, control, promoting and/or regulatory elements operably linked thereto.

25. The [A] vector according to claim 24 wherein said carrier is salmon sperm DNA.

26. The [A] vector according to claim 24 wherein said carrier is viral DNA.

27. The [A] host cell transfected with a DNA construct according to [any one of] claim[s 7 to] 22.

28. A host cell transfected with a vector according to claim 23.

29. A host cell according to claim 27 or 28 being a eukaryotic or yeast cell.

30. The [A] host cell according to claim 29 being a mammalian hemopoietic cell, fibroblast, epithelial cell, or lymphocyte.

31. The [A] host cell according to claim 27 wherein said eukaryotic cell is the baby hamster

kidney (BHK-21) cell line or the Chinese hamster ovary (CHO) cell line.

47. A pharmaceutical composition comprising as active ingredient a therapeutically effective amount of expression vectors according to any one of claims 23 to 26 or of transformed host cells according to any one of claims [27 to] 30 and 31.

48. A method of producing a recombinant therapeutic or industrially or agriculturally applicable protein comprising the steps of:

a) providing a DNA construct according to [any one of] claim 7 [to 26] or an expression vector according to [any one of] claim[s] 23 [to 26] wherein said gene encodes said protein;

b) transfecting a host cell with a DNA construct or expression vector provided in (a) to give a host cell capable of expressing said protein in substantial amount; and

c) culturing cells obtained in (b) under suitable culture conditions; and

d) isolating said protein from the cell culture obtained in (c).

49. A method of producing a recombinant therapeutic or industrially or agriculturally applicable protein comprising the steps of:

a) providing host cells transfected with a DNA construct according to [any one of] claim 7 [to 22] or an expression vector according to [any one of] claim[s] 23 [to 26] wherein said gene encodes said protein, which are capable of expressing said protein in substantial amount;

b) culturing cells provided in (a) under suitable culture conditions; and

c) isolating said protein from the cell culture obtained in (b).

#30/Dec.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Kaempfer et al.		
Serial No.	:	09/801,371	Examiner:	Whiteman, B.
Filed	:	March 7, 2001	Group Art Unit:	1635
For	:	REGULATION OF GENE EXPRESSION THROUGH MANIPULATION OF mRNA SPLICING AND ITS USES		

DECLARATION UNDER 37 C.F.R. §1.132

I, Raymond Kaempfer, hereby declare that:

1. I am one of the inventors of the invention set forth in the above-identified patent application.

2. I am a microbiologist, molecular biologist and expert in the fields of mRNA structure and function, translational control of eukaryotic gene expression and molecular biology of immunoregulation, especially post-transcriptional control of human cytokine gene expression. Attached hereto as **Exhibit A** is my *curriculum vitae*.

3. I have reviewed the subject patent application, which I have been advised is the U.S. Patent Application No. 09/801,371, which is a national phase application of PCT/IL99/00483, which designated the United States and was filed on September 6, 1999, published in English, and claims the benefit of IL 126112 filed on September 6, 1998 and IL 126757, filed on October 26, 1998.

I have also reviewed the pending Group I claims (claims 1-31 and

47-49) and the November 4, 2002 non-Final Office Action, in which the Examiner rejected claims 1-3 and 7-11 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors had possession of the claimed invention. More specifically, the Examiner contends that these claims are readable on a genus of a *cis*-acting nucleotide sequence, which is capable of rendering the removal of introns from a precursor transcript, dependent upon activation of a *trans*-acting factor. However, according to the Examiner, the specification does not provide an adequate written description of a "genus" of *cis*-acting nucleotide sequence, for example, any description of specific biochemical or molecule structure (e.g., nucleotide sequence) of a representative number of species of such *cis*-acting nucleotide sequences. The Examiner thus states that the disclosure provides sufficient support only for SEQ ID NO: 1 and 2, but not for the "generic" *cis*-acting nucleotide sequences.

In addition, the Examiner rejects claims 2, 8-9 and 11 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter of the invention because the statement in claim 2 : "A *cis*-acting nucleotide sequence" is indefinite because it does not point out which sequence a *cis*-acting nucleotide sequence is referred to in the claim. Claim 2 is not a multi-dependent claim and therefore the dependent claim should state "The *cis*-acting nucleotide sequence". Still further, the statement in claims 8-9 and 11, "A DNA construct according to claim 7" is indefinite because it does not point out which construct a DNA construct is referring to in the claim. Claims 8-9 and 11 are not multi-dependent claims and therefore the dependent claim should state "The DNA construct according to claim 7".

Still further, the Examiner contends with respect to using a trans-acting factor in claims 1-3 and 7-11, that the specification only supports the use

of PKR as the *trans*-acting factor and not any other RNA-activated protein kinases capable of phosphorylating the alpha-subunit of eIF2.

The Examiner further rejects claim 1 under 35 U.S.C. § 102(b) as being anticipated by Pennica et al. (Nature, Vol. 312, 1984, pp. 724-729), and claims 1-3 and 7-10, under 35 U.S.C. § 102(b) as being anticipated by Jarrous et al. (Mol. Cell. Biol., Vol. 16, 1996, pp 2184-2822).

Furthermore, I am aware of the state of the art prior to September 7, 1998, regarding nucleotide sequence of the TNF-α cDNA and the selective inhibition of splicing of the TNF-α mRNA by 2-Aminopurine (2-AP).

4. I understand the issues raised by the Examiner in the November 4, 2002 non-Final Office Action to be the following:

(A) First, whether the specification provides sufficient description to allow one skilled in the art to identify and use any *cis*-acting nucleotide sequence that activates PKR and thereby regulates mRNA splicing dependent on PKR and sensitive to 2-AP.

The Examiner points out that the disclosure only provides sufficient description for SEQ ID NO: 1 and 2, but however, does not provide sufficient description of a genus of *cis*-acting nucleotide sequence. More specifically, the Examiner states that the as-filed specification does not provide an adequate written description of a representative number of species of *cis*-acting nucleotide sequences.

In my opinion, the subject specification does allow one skilled in the art to conclude that a 2-AP responsive element (2-APRE) in a given gene may be capable of rendering the removal of introns from a precursor transcript encoded by a gene, dependent upon activation of a *trans*-acting factor. Such 2-APRE may

function as a *cis*-acting sequence which renders splicing dependent on PKR (as the *trans*-acting factor) and sensitive to 2-AP. Moreover, the disclosure does provide to one skilled in the art, methods and assays of screening for *cis*-acting elements in a given gene, which respond to 2-AP (for example, Example 2 of the present application). The specification further provides methods for analyzing a PKR- dependent splicing (using the dominant negative mutant of PKR, PKRΔ6), and methods for analyzing activation of PKR by such elements (Example 5, phosphorylation assay).

Thus, the Application as filed provides the teaching to enable one of ordinary skill in the art using routine experimentation, not undue experimentation, to identify *cis*-acting elements other than the elements of SEQ ID NO: 1, and 2, for use in the present invention.

Further support for such conclusion is directly provided by recent experimental data established in my laboratory [attached as **Exhibit B** (Figs. 1-10), and further discussed below] indicating the identification of two other novel 2-APRE sequences derived from the human β-globin and the human IFN-γ genes. These novel *cis*-acting elements, render splicing dependent on the activation of PKR and sensitive to inhibition by 2-AP.

These recent experiments further demonstrate the general mutual role of 2-APRE (as a *cis*-acting element) and PKR (as a *trans*-acting element) in regulation of gene expression, specifically, regulation of splicing.

(B) Second, whether the specification provides any essential properties defining such genus *cis*-acting element.

The Examiner states that it is apparent from the state of the prior art exemplified by Ngo et al. (The protein folding problem and tertiary structure prediction, Birkhauser Boston, 1994, pp 491-494) and Chiu et al. (Folding and Design, Vol. 3, 1998, pp 223-228) that the description of the primary sequence is

an essential element required for an adequate description of a representative number of species as embraced by the claimed genus of *cis*-acting nucleotide sequence and is neither described sufficiently in the specification nor conventional in the prior art.

Recent experiments conduced in my laboratory [**Exhibit B**] have shown that by using the methods disclosed in the present invention, two additional 2-APRE elements derived from different genes have been identified. As discussed below, both novel elements share no structural feature (sequence homology) with the *cis*-acting element of the TNF-α gene found by the invention. Therefore, a description using functional features is the appropriate and adequate disclosure for such elements.

5. In support of my opinion in (A) above, I point out that the subject specification has shown for the first time a novel splicing control model, based on a concretely defined novel *cis*-acting nucleotide sequence which is the 2-APRE and a defined *trans*-acting element which is the PKR. As indicated above, the disclosure further teaches the skilled person how to identify such *cis*-acting elements, and analyze their splicing regulatory properties (e.g., PKR-dependent, 2-AP sensitive and activator of PKR).

Later studies conducted in my laboratory, analyzed the function of the 2-APRE of TNF-α gene. These experiments are demonstrated by **Exhibit B** (Figs. 1-10). To facilitate analysis of 2-APRE function, we constructed a TNF-β gene carrying the TNF-α 2-APRE in its 3'-UTR, thus rendering splicing completely dependent on activation of PKR. This allowed us to take advantage of the exquisite dependence of the efficiency of TNF-β mRNA expression on the 2-APRE, shown in Fig. 1, without the confounding effect of the TNF-α AU-rich

element (ARE). As seen in Fig. 1, the presence of the 2-APRE dominates the level of expression, which is tenfold higher when this element is present, whether or not the TNF-α or TNF-β ARE is included. The TNF-α ARE is an effective instability determinant, with a number of AUUUA repeats, whereas the weak TNF-β ARE harbors just one such motif (Bakheet et al., 2001, **Exhibit D**), predicting a relatively greater stability of TNF-β pre-mRNA and mRNA, as is indeed observed.

The TNF-α 2-APRE thus is a *cis*-acting element that activates PKR and thereby renders mRNA splicing far more efficient. To show that this property is not unique to the 2-APRE sequence, we first constructed a splicing reporter gene carrying the first part of the human β-globin gene fused in-frame to green fluorescent protein (GFP) expressed under the tetracycline promoter. Excision of β-globin intron 1 yields on translation a fusion protein that is green fluorescent. Failure to splice out the intron precludes the production of this fusion protein (Fig. 2A). A control construct lacking the intron expresses the fluorescent fusion protein without dependence on splicing (Fig. 2B). As seen in Fig. 3, fluorescence was induced by tetracycline in cells stably transfected with either gene. However, the intron-containing construct failed to yield fluorescence when 2-AP was present or when the cells had been co-transfected with dominant-negative mutant PKRΔ6 (Fig. 3). By contrast, the control construct yielded fluorescence in each case. Therefore, splicing of β-globin intron 1 requires the activation of PKR. The β-globin sequence thus contains a novel element that is a functional 2-APRE.

The β-globin sequence shares another property with the 2-APRE from the human TNF-α gene, that is, it strongly activates PKR *in vitro*, inducing eIF2α phosphorylation (Fig. 4). Activation of PKR was observed when intron 1 was present in the β-globin RNA transcript but not when it was absent. Indeed,

a transcript truncated 51 nt into intron 1 activated PKR most effectively. Hence, the human β-globin gene, which does not share sequence homology with the human TNF-α gene, nevertheless contains a powerful PKR activator element and this element requires the presence of at least part of the intron 1 sequence. That the human β-globin exon 1-intron 1-exon 2 sequence is a functional 2-APRE for splicing in intact cells is shown in Fig. 5, where the β-globin sequence was inserted into the TNF-β 3'-UTR instead of the 2-APRE (Fig. 5C). This insertion caused splicing of the encoded mRNA to become highly sensitive to inhibition by 2-AP (Fig. 5A), the ratio of unspliced pre-mRNA to spliced mRNA increasing about twentyfold when 2-AP was present.

The human β-globin exon 1-intron 1-exon 2 RNA served as efficient substrate for splicing *in vitro* in nuclear extract from cells (Fig. 6, lanes 2 and 3 from left). The splicing reaction was blocked in the presence of anti-PKR antibody, with antibody against SC-35, a known splicing component, serving as positive control (Fig. 6). Therefore, splicing of β-globin exon 1-intron 1-exon 2 RNA requires PKR. That active PKR is needed is seen from the *in vitro* splicing analysis of Fig. 7, where splicing was blocked completely by a mAb directed against the catalytic domain of PKR. Thus, we have identified a novel 2-APRE-like element in the human β-globin gene that renders splicing of intron 1 dependent on the activation of PKR, both in cells and *in vitro*, and strongly activates PKR *in vitro*. Further evidence that the β-globin element acts in this manner is provided by data in Fig. 8. *In vitro* splicing was inhibited by antibody against phosphorylated PKR (Fig. 8A), showing again that activated PKR is present in the splicing reaction and is required for splicing of the β-globin RNA substrate. Moreover, *in vitro* splicing was inhibited by purified recombinant dominant-negative mutant PKRΔ6 protein, directly showing that active PKR is required for the β-globin RNA splicing reaction (Fig. 8B).

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That the requirement for PKR in splicing is specific for β-globin RNA follows from data in Fig. 9. Splicing of the β-globin RNA substrate was blocked by mAb against phosphorylated PKR whereas splicing of adenovirus Minx RNA was insensitive.

We have thus shown that at least one other gene, not a cytokine gene, is regulated at mRNA splicing by a sequence element that activates PKR and renders splicing dependent on the activation of PKR. This result particularly confirms that the application as filed teaches the general concept of splicing control by a *cis*-acting element (2-APRE) and a *trans*-acting element (PKR), together with functional assays which allow one of ordinary skill in the art to identify additional elements which share the same functional properties but however do not necessarily share sequence homology.

This finding suggested that the basic requirement for splicing control by a 2-APRE-like sequence could be fulfilled by inserting any RNA element that activates PKR. To substantiate this novel concept, we have taken advantage of our discovery that the 5'-terminal 203 nt fragment of human interferon-gamma (IFN-γ) mRNA surprisingly contains a powerful PKR activator element and that this element is an RNA pseudoknot (Ben-Asouli et al., 2002, **Exhibit C**). By strongly activating PKR in the vicinity of the mRNA molecule, the pseudoknot causes phosphorylation of eIF2α and inhibition of translation. Mutations that impair the ability to activate PKR enhance translation efficiency of IFN-γ mRNA, *d1* being such a mutation (Ben-Asouli et al., 2002, **Exhibit C**). When DNA encoding this 203-nt IFN-γ mRNA fragment was inserted into the TNF-β gene in its 3'-UTR, at the same position as was used for the 2-APRE or β-globin gene sequences, this insertion significantly enhanced the expression of the chimeric TNF-β mRNA in transfected cells and rendered splicing of the mRNA sensitive to inhibition by 2-AP, properties typical of the 2-APRE, as seen from a greater accumulation of unspliced RNA at the

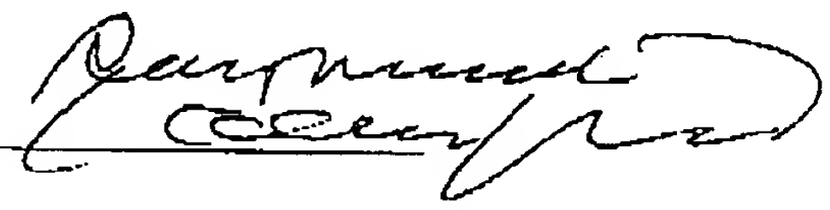
expense of spliced mRNA. By contrast, *d1* mutant IFN-γ sequence had a weaker effect (Fig. 10 of **Exhibit B**), in line with the fact that the *d1* mutation leaves only a low residual ability to activate PKR (Ben-Asouli et al., 2002, **Exhibit C**).

6. In support of my opinion in (B) above, I point out that recent experiments performed in my laboratory (**Exhibit B** and discussed above) have indicated that the 2-APRE sequence of the TNF-α gene is by no means unique in rendering splicing dependent on the activation of PKR and sensitive to inhibition by 2-AP. These properties are not vested in one particular RNA sequence *per se* but must rely on a broader scope of structural features in the RNA that can be achieved starting from quite distinct sequences, for example, from unrelated elements in human β-globin and IFN-γ genes. Therefore, in my opinion, defining such *cis*-acting element by using nucleotide sequence (e.g. SEQ ID NO: 1 and 2 of the present application) as structural properties would be limited and not suitable. Thus, appropriate definition of such novel and inventive splicing regulatory elements should be based on functional properties (e.g. capable of rendering removal of introns from a precursor transcript in a PKR dependent and 2-AP sensitive manner and capable of activating PKR).

7. In my opinion, in view of the methods and examples publicly available as of the priority date of the subject application, i.e. September 7, 1998, and the teachings set forth in the subject application and in the attached recent experimental data obtained in my laboratory, one of skill in the art would have been able, without undue experimentation, to screen for *cis*-acting elements capable of controlling splicing, prepare expression vectors containing such regulatory elements and use these elements in control of expression of any desired gene.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing thereon.

Dated: May 4, 2003

Raymond Kaempfer 

RAYMOND KAEMPFER

Education

1940	Born March 1 in The Hague, The Netherlands
1958-61	BS in Chemistry, University of Leiden, The Netherlands
1961-65	PhD in Microbiology, Massachusetts Institute of Technology, Cambridge, Massachusetts
1965-66	Van Leer Fellow, Weizmann Institute of Science
1966-68	Fellow of the Jane Coffin Childs Memorial Fund for Medical Research, Harvard University, Cambridge Massachusetts
1968-69	NIH Special Fellow, Harvard University

Academic Appointments

1978-now	Professor of Molecular Biology, The Hebrew University-Hadassah Medical School, Jerusalem
1974-78	Associate Professor of Molecular Biology, The Hebrew University-Hadassah Medical School, Jerusalem
1969-74	Assistant Professor of Biology, Harvard University
1967-69	Tutor of Biology, Harvard College
1965	Research Associate, Massachusetts Institute of Technology
1965	Instructor of Microbial Genetics, Massachusetts Institute of Technology

Distinctions

1964-65	Woodrow Wilson Fellow, Massachusetts Institute of Technology
1983	Appointed to the Dr. Philip M. Marcus Chair in Molecular Biology and Cancer Research of The Hebrew University

Elected Memberships

1982-now Member, European Molecular Biology Organization (EMBO)

Visiting Appointments

1976 Visiting Professor, Laboratoire de Biochimie Virale
Fondation Curie-Institut du Radium, Paris (one month)
1977 Visiting Professor, Laboratoire de Biochimie Virale
Fondation Curie-Institut du Radium, Paris (one month)
1978 Research Consultant, Department of Biochemistry, Pahlavi
University Medical School, Shiraz, Iran (one month)
1979-80 Visiting Scientist, Laboratory of Molecular Biology, State
University of Gent, Belgium
1980 Staff Member, NATO Advanced Study Institute on Protein
Biosynthesis in Eukaryotes, Maratea, Italy
1981 Visiting Exchange Professor, Institute of Virology, University
of Rome and Laboratory of Cell Biology, National Research
Council of Italy (one month)
1985 Visiting Exchange Scientist in Biotechnology, Japan (one month)
1990 Visiting Professor of Molecular Biology, GBF-TU,
Braunschweig, Germany (one month)

Appointments in Biotechnology

1984-91 Founder and Chairman, Graduate Program in Biotechnology, The
Hebrew University
1984-91 Member, Steering Committee on Biotechnology
The Hebrew University
1984-88 Member, National Subcommittee on Manpower and Infrastructure
in Biotechnology, Ministry of Science and Development, Israel

Research Interests

- mRNA structure and function
- Translational control of eukaryotic gene expression
- Molecular biology of immunoregulation, especially post-transcriptional control of human cytokine gene expression
- Molecular biology of Th1 cytokine gene activation by superantigens

Publications

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Exhibit B

Figure 1. Contributions of 2-aminopurine response element (2-APRE) and downstream 3'-UTR sequences to human TNF-β mRNA expression. BHK-21 cells were transfected with pSV_2CAT and TNF-β gene construct. Four TNF-β constructs are shown, from top: the intact TNF-β gene [TNF-β]; truncated to remove the ARE [TNF-β(Δ3'UTR)]; carrying part of the TNF-α 3'-UTR including its 2-APRE and ARE [TNF-β(3'UTRα)]; and truncated but carrying only the 2-APRE [TNF-β(3'UTR-αEP)]. Magenta color denotes TNF-α 2-APRE; An(α), TNF-α 3'-polyadenylation sequence; An(β), TNF-β 3'-polyadenylation sequence, ex, exon; TNF-β introns are shown in light green color. Expression of spliced TNF-β mRNA and of CAT mRNA was quantitated by RNase protection analysis (top) and their ratio is plotted (bottom). The TNF-β intron 3-exon 4 antisense RNA probe used for RNase protection analysis detects unspliced RNA (637 nt band; not shown) and spliced mRNA (571-nt band). The 2-APRE enhances expression of TNF-β mRNA by about tenfold.

Figure 2. Reporter construct for splicing of human β-globin first intron. Top, in the βG(ex1-in-ex2)GFP construct the tetracycline promoter (*p*Tet) is abutted to human β-globin (βG) genomic exon 1-intron 1-exon 2 sequence fused in-frame to green fluorescent protein (GFP). Removal of the βG intron (Splic +) results in an intact open reading frame and expression of β-globin/GFP fusion protein (Prot) whereas lack of splicing (Splic -) leads to retention of the intron which encodes a translation stop codon in all 3 reading frames, resulting in premature termination and lack of fluorescence. Bottom, βG(ex1-ex2)GFP control construct, lacking the βG intron, yields β-globin/GFP fusion protein independent of splicing.

Figure 3. Activation of PKR is required for splicing of the human β-globin first intron. BHK-21 cells were stably transfected with βG(ex1-ex2)GFP control construct (top) or βG(ex1-in-ex2)GFP construct (bottom). Fluorescence was determined for equal numbers of cells by confocal fluorescence microscopy (red color) before (-) or 3 h after induction of the cells with tetracycline (+). Where indicated, 20 mM 2-AP was added at the time of induction. Where indicated, the cells were transfected with pPKRΔ6 which expresses a dominant negative mutant of PKR and induced with tetracycline 48 h later.

Figure 4. Activation of PKR by human β-globin RNA transcripts harboring first intron sequence. Human β-globin genomic exon 1-intron 1-exon 2 sequence or control β-globin genomic sequence lacking intron 1 was transcribed from the SP6 promoter using a cut plasmid (Promega) and following the manufacturer's instructions. A 499-nt transcript was transcribed from the full DNA template and a 189-nt transcript after first digesting the DNA with *Nsp*I. A 367-nt transcript was obtained from the β-globin cDNA plasmid pSP64.βglo after first digesting the DNA with *BamH*I. The RNA product was rigorously purified to remove any contaminating dsRNA (Osman et al., 1999). Activation of PKR was assayed in the ribosome fraction of rabbit reticulocyte lysate in the presence of the indicated amounts of poly rI:rC (dsRNA) or of β-globin transcript carrying intron 1 (499 nt), lacking intron 1 (367 nt), or carrying intron 1 and truncated 51 nucleotides after the

start of the intron (189 nt). Phosphorylation of PKR (68 kDa) and eIF2α (38 kDa) bands is indicated in the autoradiogram obtained after SDS-PAGE.

Figure 5. Human β-globin genomic exon 1-intron 1-exon 2 sequence is a functional 2-APRE. The 499-bp β-globin genomic exon 1-intron 1-exon 2 sequence was inserted into the TNF-β 3'-UTR at the same position as the 2-APRE in TNF-β(3'UTR-αEP) in Fig. 1 (map: Fig. 5C). (A) Expression of unspliced RNA (637 nt band) and spliced mRNA (571-nt band) was quantitated in transfected BHK-21 cells by RNase protection analysis with a TNF-β intron 3-exon 4 antisense RNA probe at the indicated times after transfection. Where indicated, 20 mM 2-AP was added to the medium at 18 h after transfection. (B) Pre-mRNA over mRNA ratio is plotted for each time point using data from (A)(triangles, without 2-AP; squares, with 2-AP). T, time.

Figure 6. In vitro splicing of β-globin intron 1 is inhibited by antibody directed against PKR. Nuclear extract from HeLa cells (Promega, Madison, Wisconsin) was incubated with splicing buffer (Promega) and ^{32}P-labeled in vitro β-globin transcript carrying intron 1 (499 nt)(cf. Fig. 4)(P), following the manufacturer's instructions. Antibody to PKR (αPKR), to SC-35 (αSC-35) or normal goat serum (NGS) (Santa Cruz Biotechnology, Santa Cruz, CA) was added where indicated, in the amounts shown. After 3 h, the splicing reaction (25 μl) was terminated and RNA was analyzed by polyacrylamide gel electrophoresis and autoradiography. Unspliced RNA, 499 nt; spliced RNA, 367 nt.

Figure 7. mAb directed against the catalytic domain of PKR blocks splicing of β-globin RNA in vitro. In vitro splicing was performed as in Fig. 6, in the absence or presence of the indicated amounts of a mAb against the catalytic domain of PKR (Santa Cruz Biotechnology).

Figure 8. mAb directed against phospho-PKR (A) and recombinant PKRΔ6 protein (B) each inhibit splicing of β-globin RNA in vitro. In vitro splicing was performed as in Fig. 6, in the absence or presence of the indicated amounts of a mAb directed against phospho-PKR (αP-PKR)(Biosource International, Nivelles, Belgium)(A) or in the absence or presence of the indicated amounts of histidine$_6$-tagged recombinant PKRΔ6 protein expressed in *E. coli* and purified from bacterial extract on a Ni column (rPKRΔ6)(B). M, size marker DNA. In (A) and (B), lane 5 is a control incubated, respectively, with αP-PKR and rPKRΔ6 buffer only, in the equivalent amount to that used for lane 4.

Figure 9. Inhibition of in vitro splicing of β-globin intron 1, but not of adenovirus Minx intron, by mAb directed against phospho-PKR. In vitro splicing as in Fig. 8 was performed in the absence or presence of the indicated amounts of a mAb directed against phospho-PKR (Biosource International)(αP-PKR), using either 499-nt unspliced β-globin RNA transcript or 226-nt unspliced adenovirus Minx (MINX) transcript (unsplic) as substrate. Spliced Minx mRNA is 104-nt in length (splic). β-globin control (bu) received αP-PKR buffer only, in the equivalent amount to the highest amount of mAb used. M,

size marker DNA. Second lane from left shows input (in) β-globin RNA (499 nt band) and Minx RNA (226 nt band) applied together.

Figure 10. Insertion of human γ-interferon mRNA pseudoknot sequence into the 3'-UTR of the human TNF-β gene renders mRNA splicing sensitive to inhibition by 2-AP. The 5'-terminal 203-nt fragment of human IFN-γ mRNA which contains a pseudoknot structure that strongly activates PKR (Ben-Asouli et al., 2002), was inserted into the TNF-β 3'-UTR at the same position as the 2-APRE in TNF-β(3'UTR-αEP) in Fig. 1, to yield TNF-β(3'UTR-IFN-γ-*wt*). A mutant form of the IFN-γ mRNA fragment that activates PKR far more weakly than *wt* was also inserted, yielding TNF-β(3'UTR-IFN-γ-*d1*). Human TNF-β gene construct lacking an insert was also transfected (TNF-β). Where indicated, 17 mM 2-AP was added to the medium at 21 h after transfection. At the indicated times after transfection, expression of unspliced RNA (340 nt band) and spliced mRNA (274-nt band) was analyzed in equal amounts of RNA extracted from transfected BHK-21 cells by RNase protection analysis, using a TNF-β intron 3-exon 4 antisense RNA probe (P) shortened at the 3' end.

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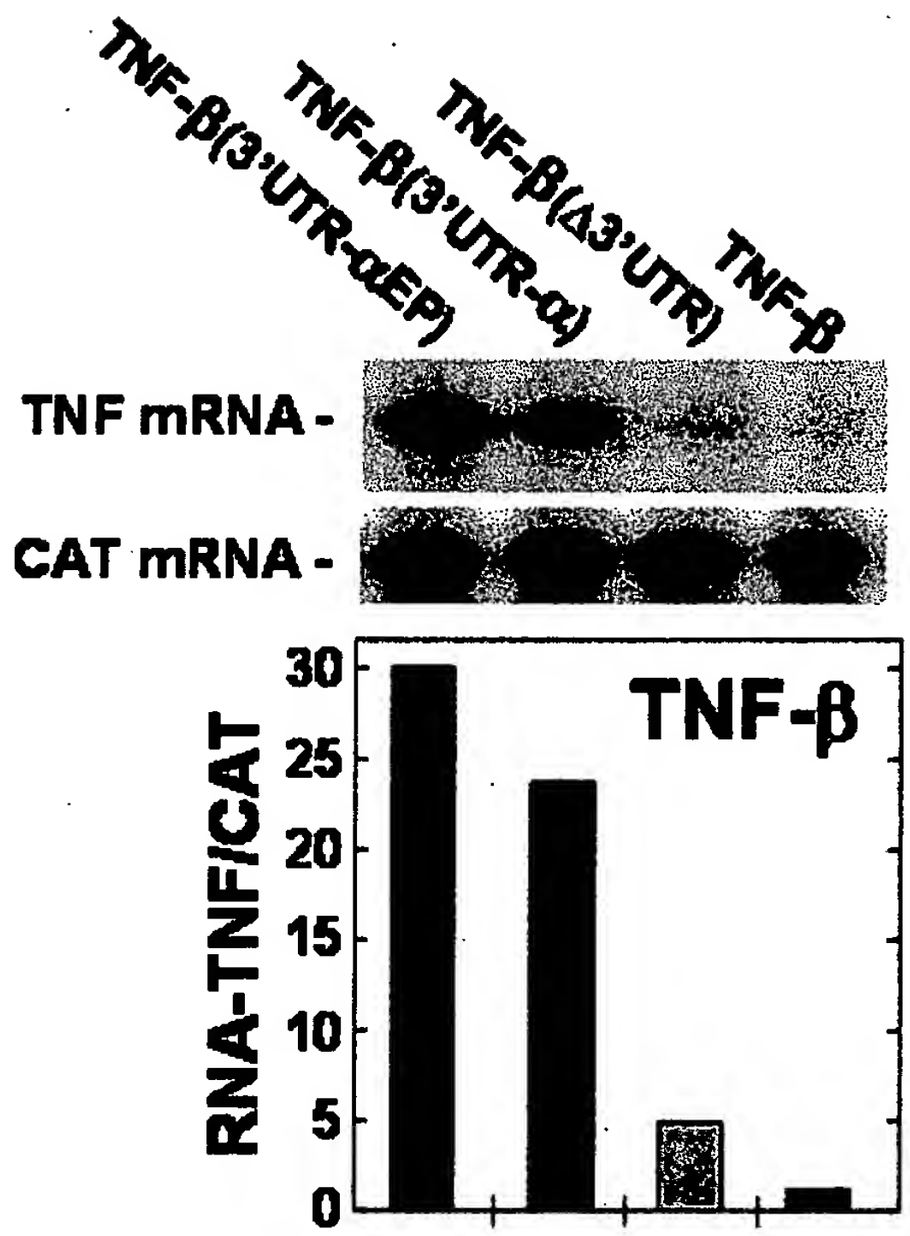
TNF-β(3'UTR-αEP)
TNF-β(3'UTR-α)
TNF-β(Δ3'UTR)
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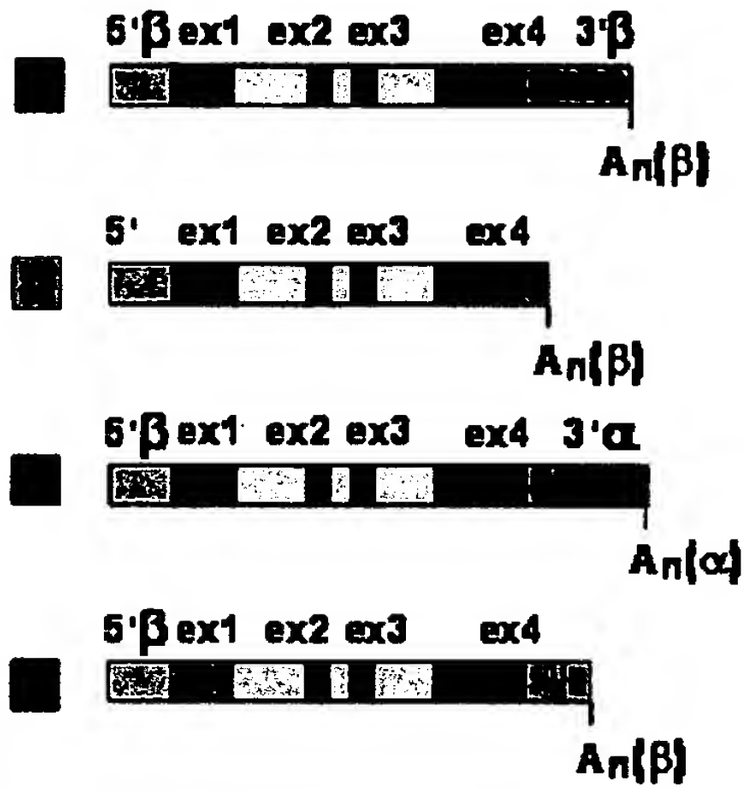
CAT mRNA -
RNA-TNF/CAT
30
25
20
15
10
5
0
TNF-β

Fig. 1


5'β ex1 ex2 ex3 ex4 3'β
An(β)
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An(β)

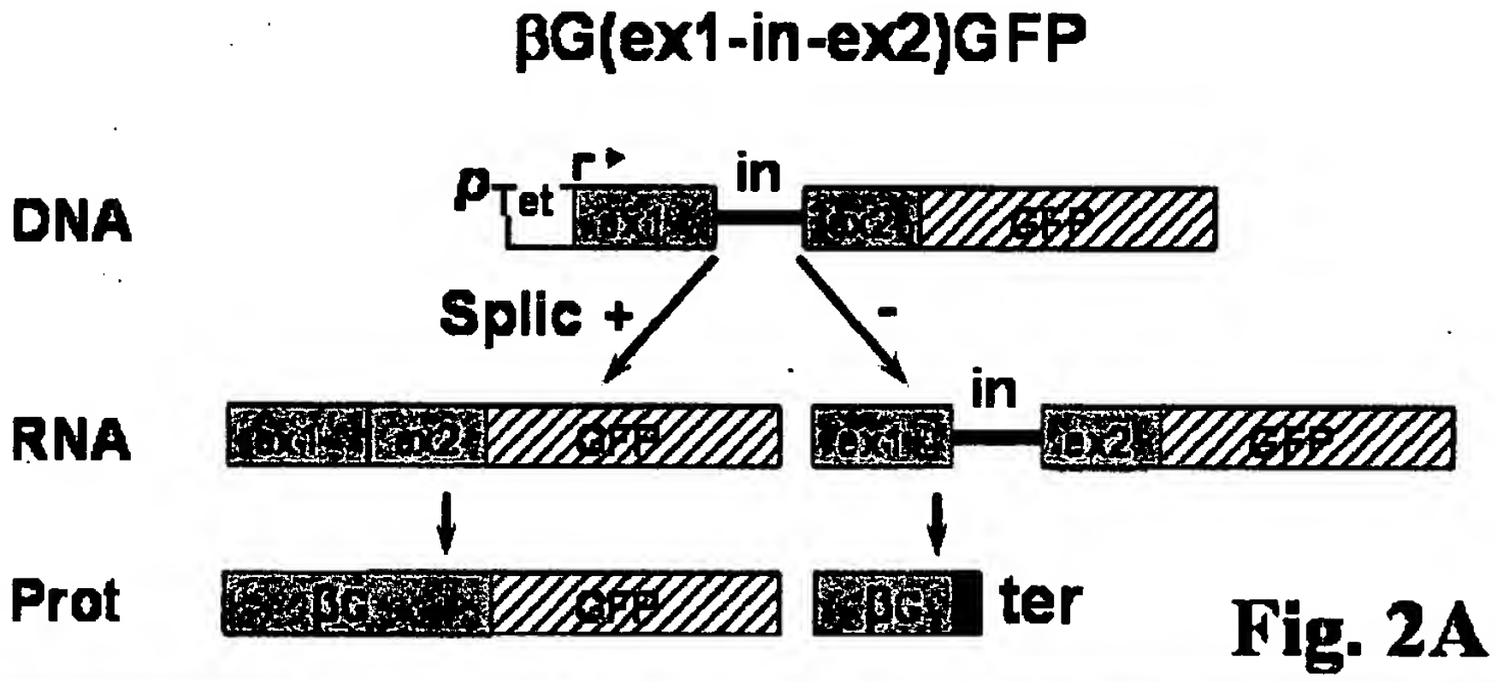
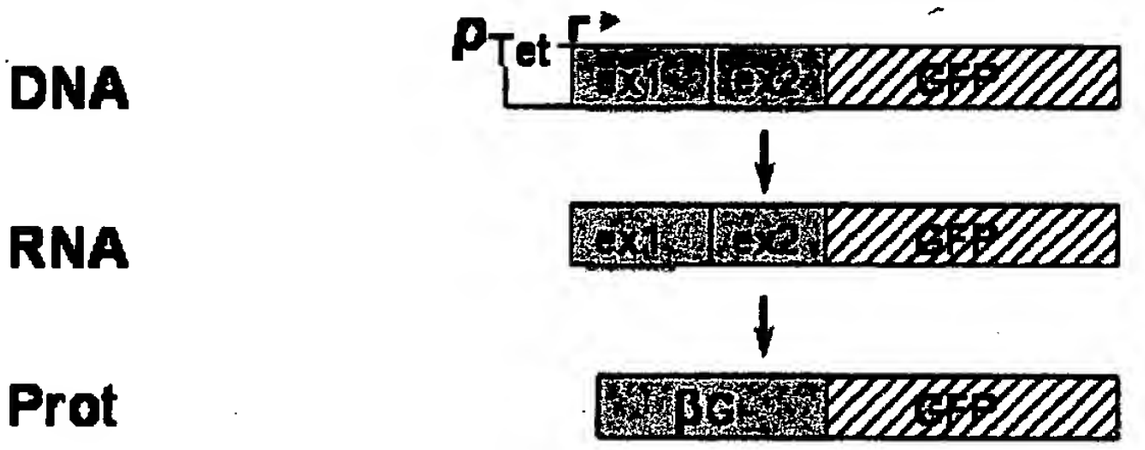


Fig. 2A

Fig. 2B



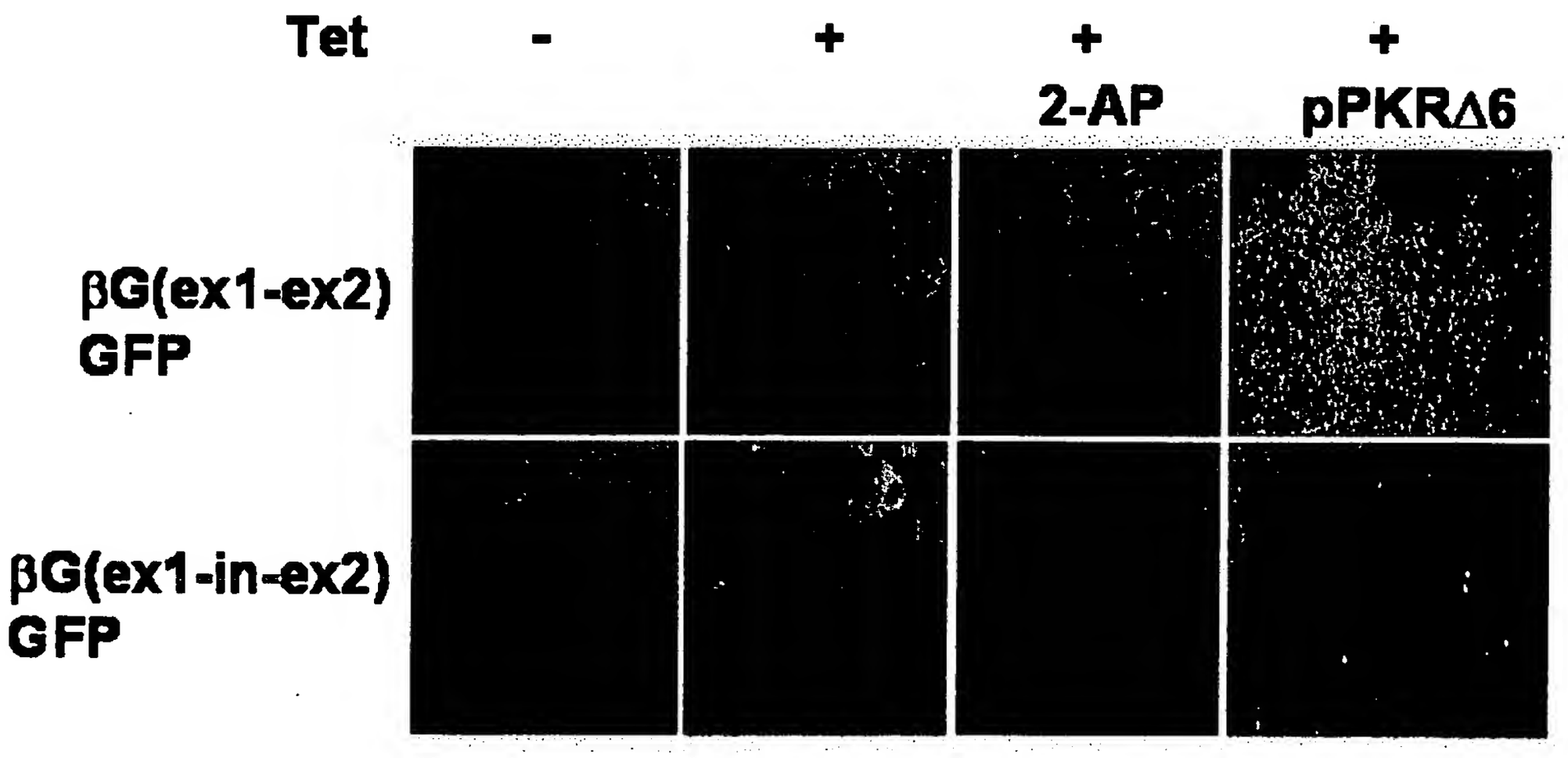


Fig. 3

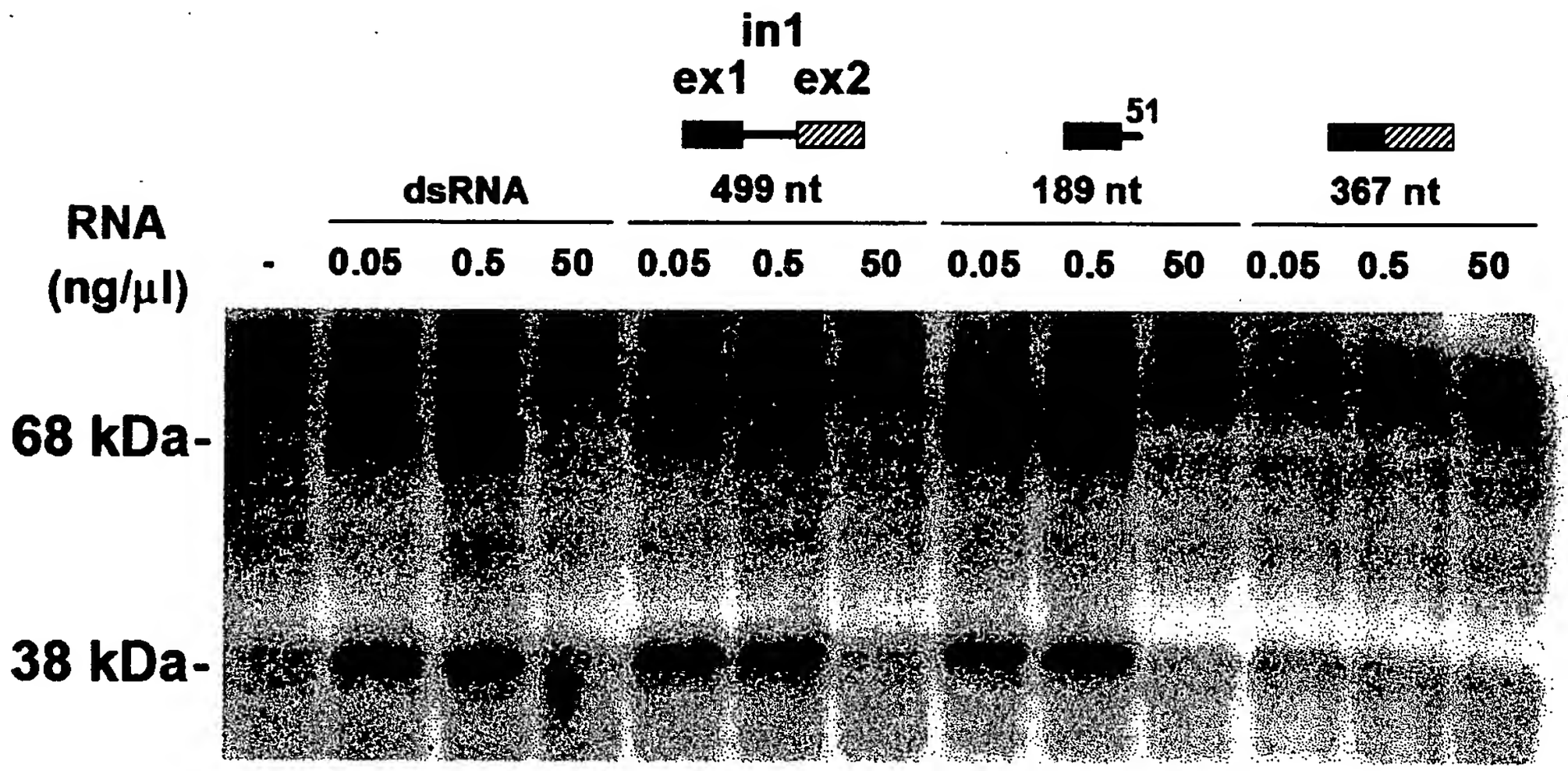

in1
ex1
ex2
51
dsRNA
499 nt
189 nt
367 nt
RNA
(ng/µl)
-
0.05
0.5
50
0.05
0.5
50
0.05
0.5
50
0.05
0.5
50
68 kDa-
38 kDa-

Fig. 4

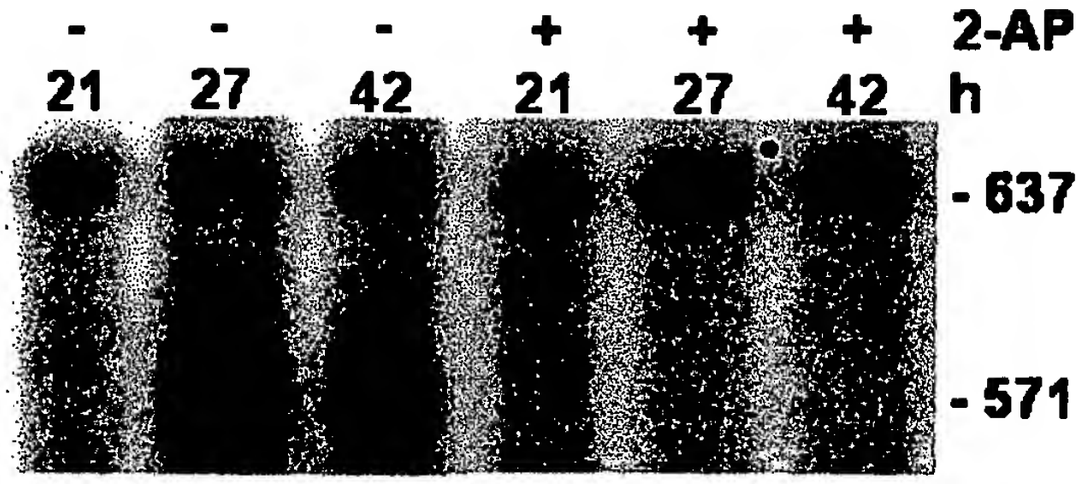
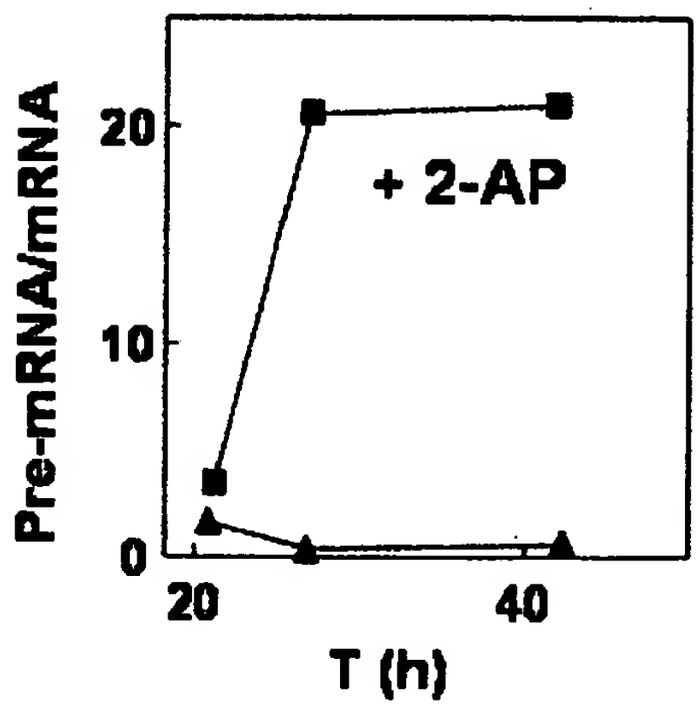


Fig. 5A



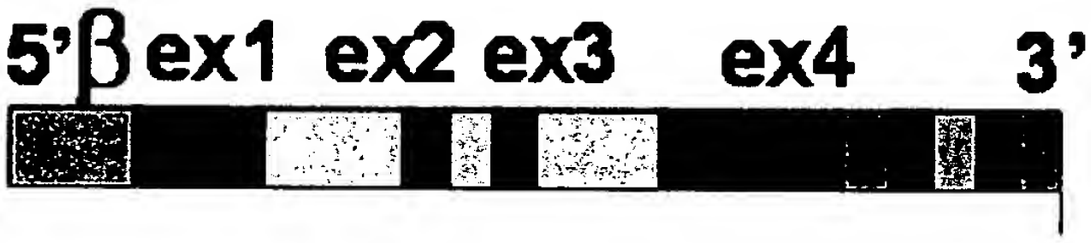


Fig. 5B

TNF-β(β-globin(ex1-in-ex2)) $A_n(\beta)$

Fig. 5C

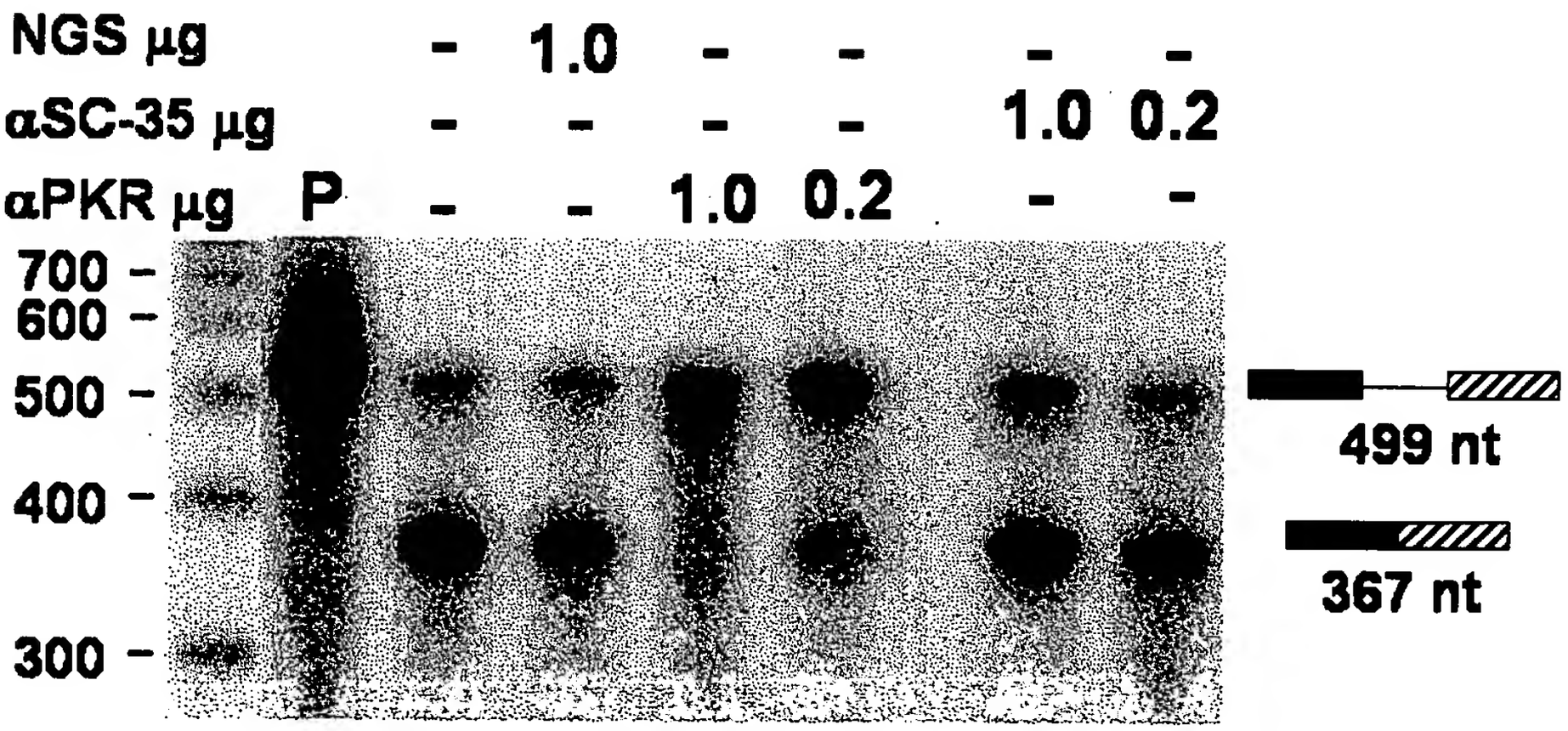


Fig. 6

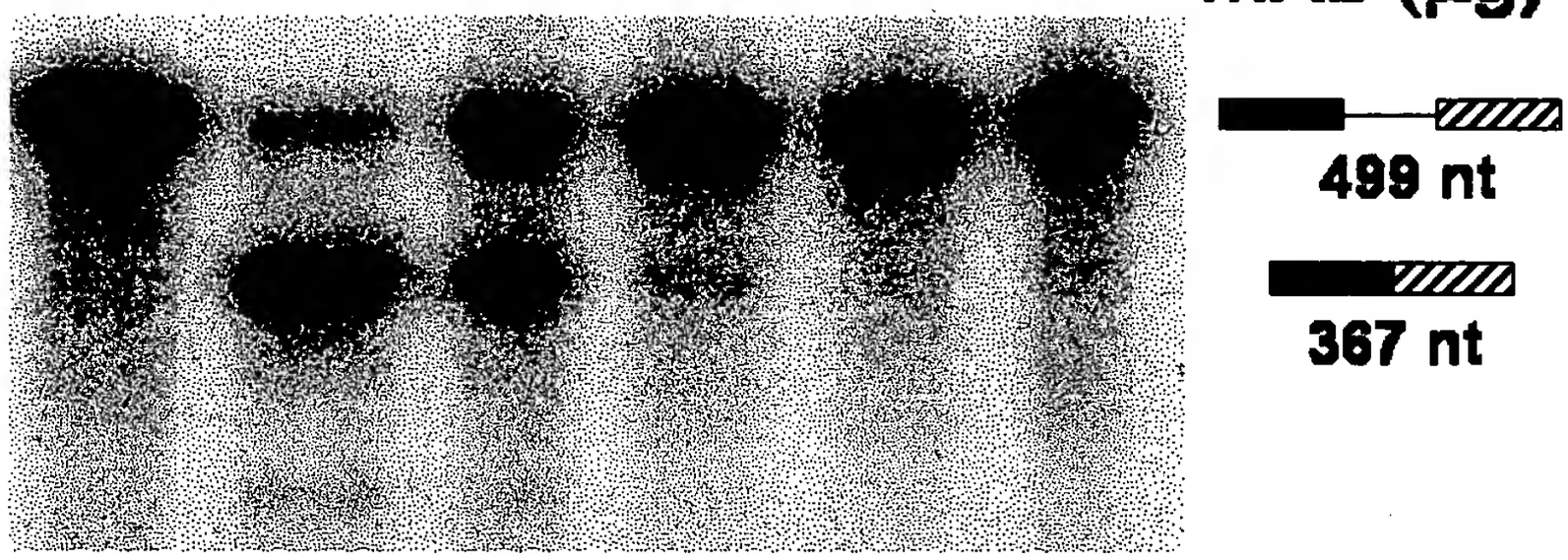
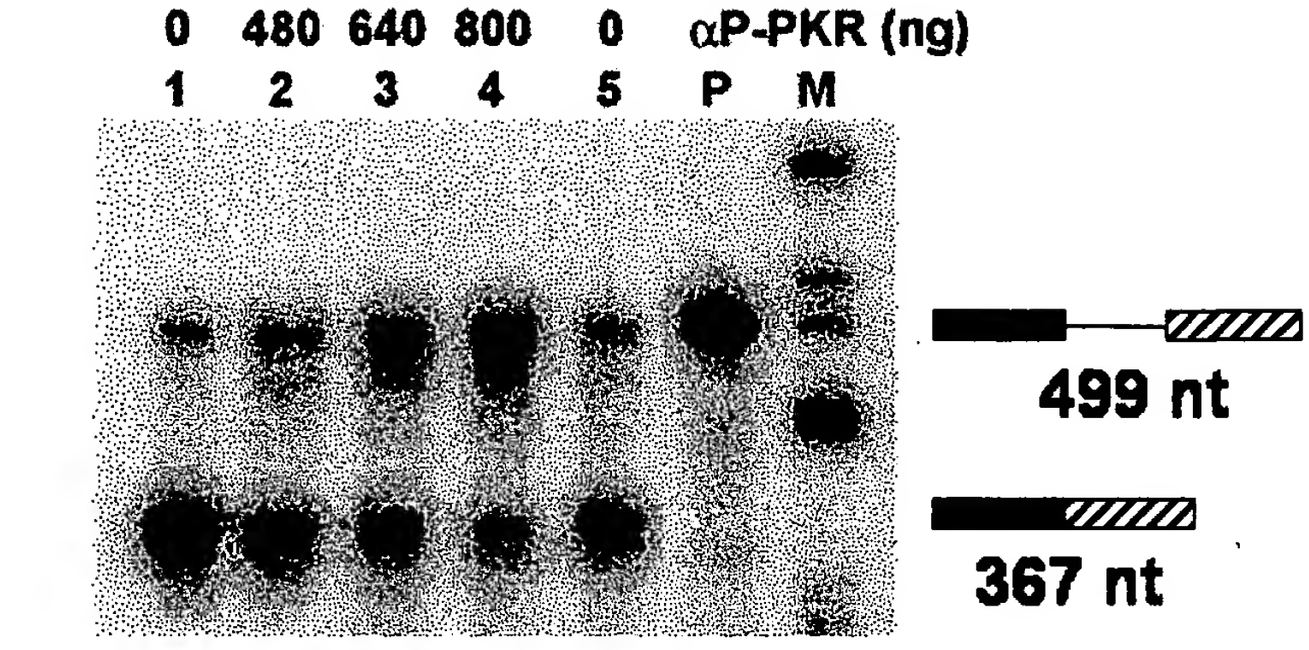


Fig. 7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1 2 3 4 5 P M
499 nt
367 nt

Fig. 8A

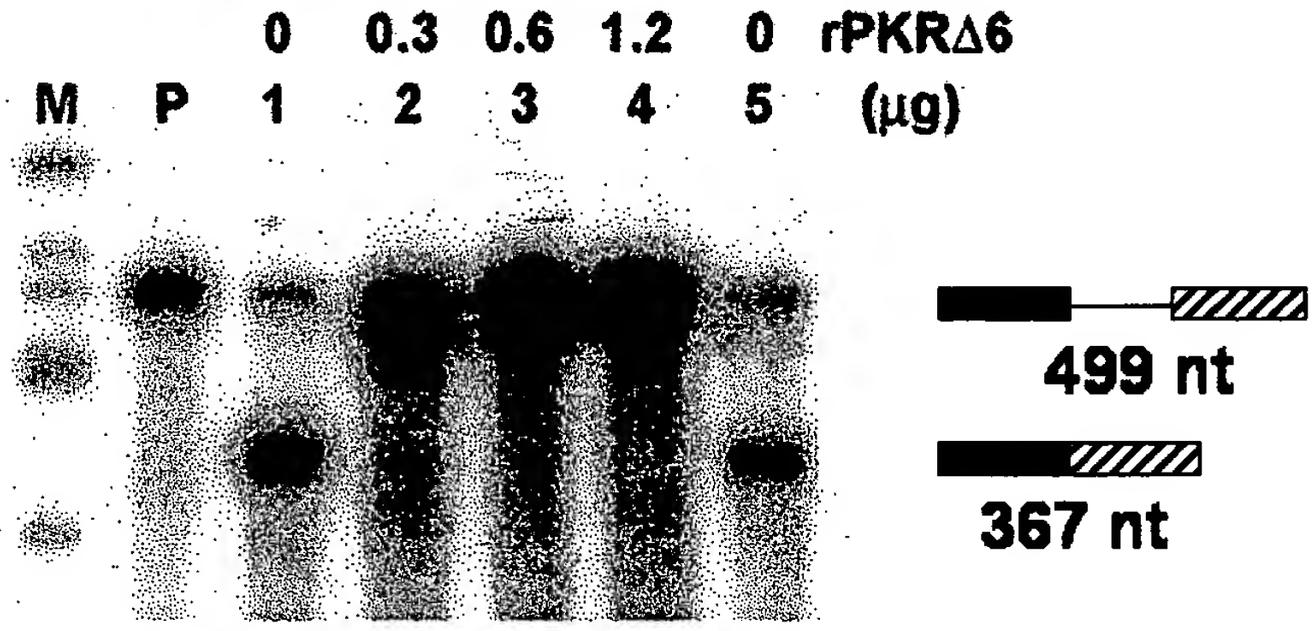
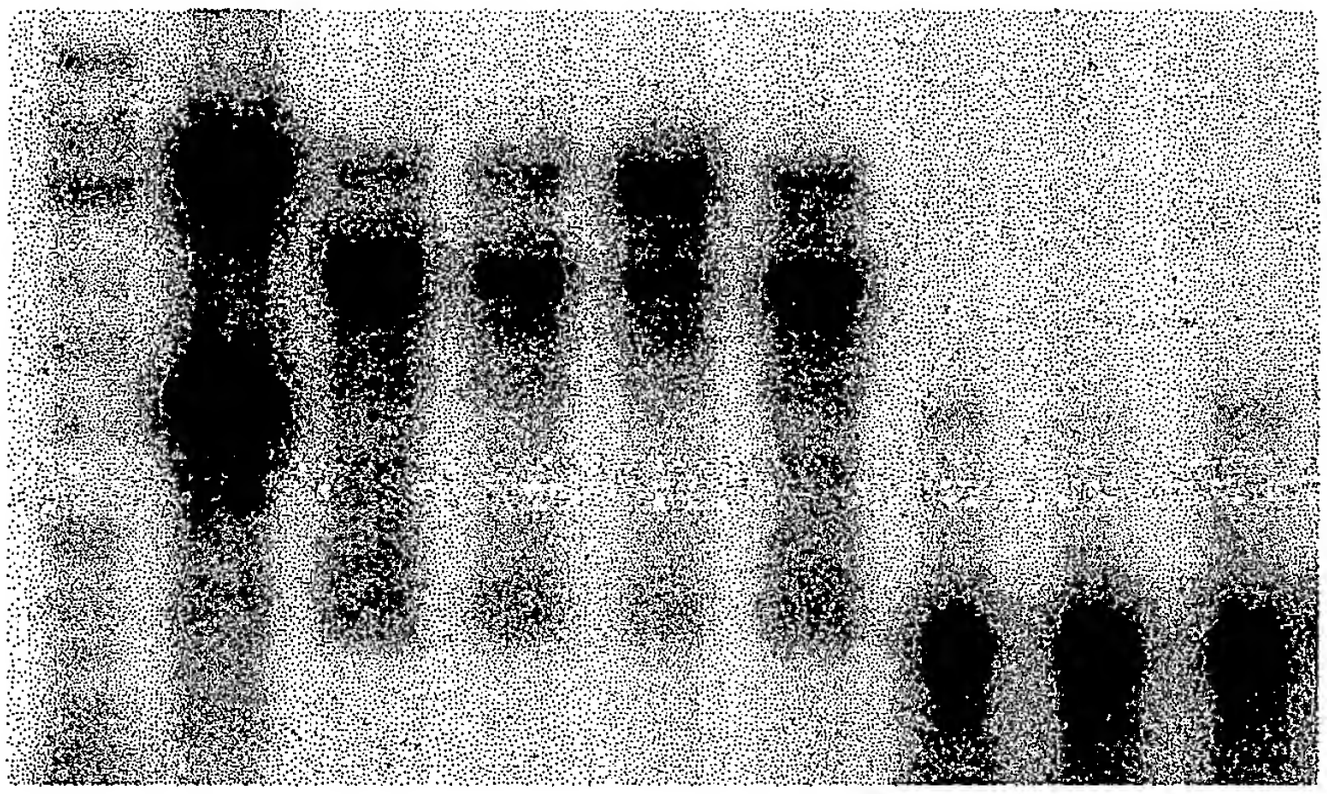

0 0.3 0.6 1.2 0 rPKRΔ6
M P 1 2 3 4 5 (μg)
499 nt
367 nt

Fig. 8B

αP-PKR (μg)	0	0.4	0.8	bu	0	0.4	0.8
β-globin RNA	+	+	+	+	-	-	-
MINX RNA	-	-	-	-	+	+	+

M in



499

367

- MINX 226 unsplic

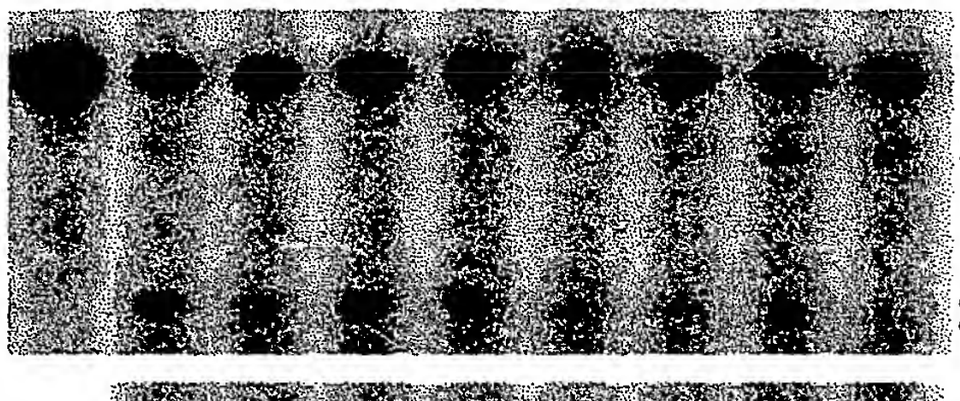
- MINX 104 splic

Fig. 9

- - - - + + + + 2-AP

P 25 30 35 40 25 30 35 40 h

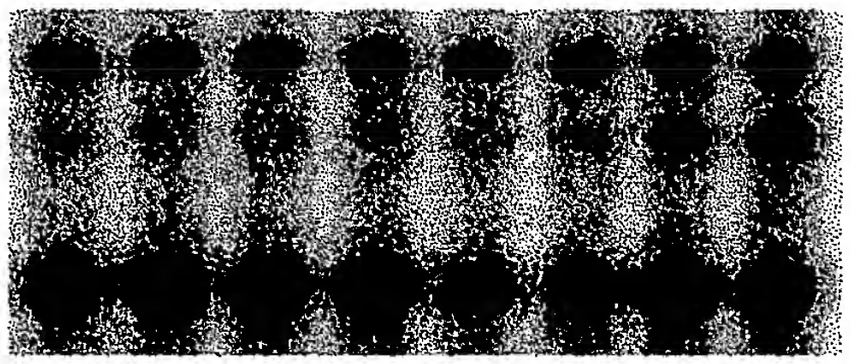
TNF-β



340

274

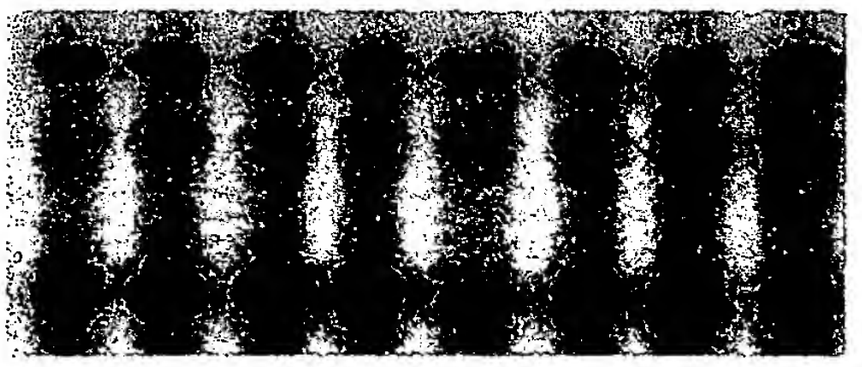
TNF-β(3`UTR-IFN-γ-*wt*)



340

274

TNF-β(3`UTR-IFN-γ-*d1*)



340

274

Fig. 10